Daz- and Pumilio-Like Genes Are Asymmetrically Localized in Pelophylax (Rana) Oocytes and Are Expressed During Early Spermatogenesis

RESEARCH ARTICLE

Daz- and *Pumilio*-Like Genes Are Asymmetrically Localized in *Pelophylax* (*Rana*) Oocytes and Are Expressed During Early Spermatogenesis

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SILVIA MARRACCI[1]*, VALENTINA MICHELOTTI[1], CLAUDIO CASOLA[2], CRISTINA GIACOMA[3], AND MATILDE RAGGHIANTI[1]

[1]Laboratory of Cell and Developmental Biology, Department of Biology, University of Pisa, Pisa, Italy
[2]Department of Biology, Indiana University, Indiana
[3]Department of Animal and Human Biology, University of Turin, Turin, Italy

ABSTRACT

In many organisms, the specification of cell fate and the formation of embryonic axes depend on a proper distribution of maternal mRNAs during oogenesis. Asymmetrically localized determinants are required both for embryonic axes and germline determination in anuran amphibians. As a model system of these processes, we have used a species complex of the genus *Pelophylax* (*Rana*), characterized by a hybridogenetic reproduction that involves events of genome exclusion and endoreduplication during meiosis in both sexes. With the aim of characterizing the still largely unknown molecular events regulating *Pelophylax* gametogenesis, we have isolated in this animal model homologues of the deleted in AZoospermia-like (*DAZl*) and *pumilio* gene families (named *RlDazl* and *RlPum1*, respectively), which encode posttranscriptional regulators. Expression pattern analysis of these genes showed that *RlDazl* is exclusively expressed in gonadal tissues, whereas *RlPum1* is expressed in both somatic tissues and gonads. In situ hybridization carried out on gonads revealed that the two transcripts were asymmetrically localized along the animal–vegetal (A–V) axis of oocytes. In particular, the *RlDazl* transcript progressively collected to the vegetal pole during oogenesis, whereas the *RlPum1* mRNA was preferentially enriched at the animal hemisphere. In adult testes, *RlDazl* and *RlPum1* were expressed in specific phases of spermatogenetic divisions as shown by immunostaining with anti-H3 phosphohistone antibody. Our results indicate that *RlDazl* and *RlPum1* represent two early indicators of oocyte polarity in this hybridogenetic vertebrate model. Additionally, *RlDazl* share with vertebrate *DAZ*-like genes a germ cell-specific expression pattern. *J. Exp. Zool. (Mol. Dev. Evol.) 316:330–338, 2011.* © 2011 Wiley-Liss, Inc.

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RNA localization represents an evolutionarily conserved strategy of translational control that plays a pivotal role in the establishment of cell polarity and/or determination of cell fate (Graindorge et al., 2006; Martin and Ephrussi, 2009). In oocytes, RNA localization has profound implications for development, by creating local concentrations of regulatory proteins that will specify different cell fates in the embryo (King et al., 2005). In both *Drosophila* and *Caenorhabditis*, genetic pathways regulating

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*Correspondence to: Silvia Marracci, Laboratory of Cell and Developmental Biology, Department of Biology, University of Pisa, Pisa, Italy.
E-mail: smarracci@biologia.unipi.it

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germ plasm assemblage are also involved in antero/posterior (A/P) axis formation of the oocyte and embryo (Zhou and King, 2004). In anuran amphibians, germ plasm assemblage at the vegetal pole contributes to the establishment of oocyte polarity by determining the animal/vegetal (A/V) axis formation and by setting up polarities in genetic information that drive cell fate during embryogenesis (King et al., 2005).

Deleted in AZoospermia (DAZ)-like and pumilio-like proteins represent a highly conserved class of translational factors implicated in the different steps of germ cell development, from determination to differentiation (Houston and King, 2000; Wickens et al., 2002). Genes belonging to *DAZ* family comprising the *boule*, *DAZ*-like (*DAZL*) and *DAZ* homologues, encode highly conserved RNA-binding proteins that are specifically expressed in germ cells and are essential for gametogenesis in metazoans (Spassov and Jurecic, 2003). In *Dazla* defective mouse, female germ cells are arrested at the prophase of meiosis I, whereas male germ cells are affected at the proliferating stage (Ruggiu et al., '97; Saunders et al., 2003). The *Xenopus XlDazl* is expressed as an RNA localized to the mitochondrial cloud of early oocytes and to primordial germ cells (PGCs; i.e. precursors of gametes) of early embryos (Houston et al., '98). Interestingly, loss-of-function studies have put in evidence a specific role of *XlDazl* in PGC migration (Houston et al., '98).

The *pumilio* family is constituted by genes encoding translational repressor proteins characterized by a carboxyl terminus pumilio homologous domain (PUM-HD), responsible for binding to specific 3′UTR mRNA sequences, reported as nanos response elements (Moore et al., 2003). The *pumilio* RNA of *Drosophila* is enriched at the posterior pole of the egg and is involved in regulation of asymmetric divisions of germline stem cells in the *Drosophila* ovary (Wickens et al., 2002). In vertebrates, two *pumilio* paralogous genes have been described (Crittenden et al., 2002; Lee et al., 2008). The *Xenopus Pumilio 2* homologue plays an important role in translational control of cyclin B1, a component of the Maturation Promoting Factor (Wickens et al., 2002; Padmanabhan and Richter, 2006). Recently, it has been shown in *Xenopus* embryo vegetal cells that the Pumilio 1 protein represses the translation of the maternal determinant xCR1 that is required for A/P patterning during *Xenopus* embryogenesis (Zhang et al., 2009). Human *PUM2* is expressed predominantly in human embryonic stem and germ cells, and the PUM2 protein colocalizes with DAZ and DAZL in germ cells, although the role of this interaction remains to be explored (Moore et al., 2003).

We have used, as an animal model, water frogs belonging to the *Pelophylax* (*Rana*) *esculentus* complex, a group of frogs containing *P. esculentus*, which has arisen by natural hybridization between the two parental species *Pelophylax ridibundus* and *Pelophylax lessonae*. *P. esculentus* hybrid represents an unusual example of fertile hybrid in vertebrates, thanks to a modified gametogenesis known as hybridogenesis (see Ragghianti et al., 2007). In the developing germ cells of the hybridogenetic hybrid *P. esculentus*, one set of the parental genomes is excluded and the remaining one endoreduplicates and then is hemiclonally transmitted to gametes (Tunner and Heppich-Tunner, '91). In this article, we describe the isolation of homologues of *DAZL* and *pumilio* genes in *Pelophylax* and analyze their expression pattern during oogenesis and spermatogenesis.

MATERIALS AND METHODS

Animals

In this study, juveniles and adults of *P. lessonae*, *P. ridibundus*, and *P. esculentus*, from near Poznan (Poland), were identified by morphometric and molecular analyses (cf. Ragghianti et al., 2007). Frogs were anaesthetized with MS222 (tricaine methanesulfonate, Sigma) and sacrificed, after which ovaries containing oocytes at different stages of development (cf. Ogielska and Kotusz, 2004) and somatic tissues were collected.

To obtain defolliculated oocytes, the ovarian tissue was incubated in 0.2% collagenase (type II, Sigma) in 0.1 M sodium phosphate pH 7.4. Testes were explanted from male individuals after they were MS222-anesthetized and sacrificed.

We followed the guidelines for animal care established by the University of Pisa.

RNA Isolation, cDNA Cloning, and Sequencing

Total RNA was isolated from adult organs using Nucleospin RNA II kit (Macherey-Nagel, Germany). A SMART cDNA library was synthesized from *P. lessonae* testis using a RACE cDNA Amplification kit (BD Biosciences). The testis library was used for isolating partial cDNA clones of *DAZL* and *pumilio*-like genes by RT-PCR with the following degenerate primers, respectively.

RlDazl
FOR 5′-TTCTCGAGTTYGTIGGIGGIATHGA-3′
REV 5′-TTAAGCTTRAAICCRTAICCYTT-3′
RlPum1
FOR1 5′-GAYCARCAYGGNTCNCGNTTYATHCA-3′
FOR2 5′-GTNATHCARAARTTYTTYGARTTYGG-3′
REV1 5′-TARTTNGCRTAYTGRTCYTTCATCAT-3′
REV2 5′-TGYTGDATNACRTARTTNCCRTAYTG-3′

To obtain complete sequences, 5′ and 3′ RACE reactions were performed using a SMART 5′/3′ RACE cDNA amplification kit (BD Biosciences) with sequence-specific oligonucleotides:

RlDazl
FOR 5′-CGGATTGATCAGCATGAAATTAAAG-3′
REV 5′-AACCAGCGCGATCAGTAATTATTTTC-3′
RlPum1
FOR 5′-TGATATGGTCCGAGAACTGGATGGGCAC-3′
REV5′-CAACCTTACGGATGCGTCGAGAGTGC-3′

In order to isolate full-length clones, the following sequence-specific primers, designed on the 5′ UTR and 3′ UTR of corresponding transcript sequences, were used:

RlDazl
FOR 5′-CTTCGGTTGTTCTAGGTTTTGTTG-3′
REV 5′-TTATTAGCCTGGGTGCAGTTTTAC-3′
RlPum1
FOR 5′-GACCTAATCCGACTCCCTCCTCCCG-3′
REV 5′-AAGAGCTACACCCTGATTCTCCCACG-3′

The PCR products were TA-cloned into pGEM-T easy vector (Promega, Italy) and sequenced by automated fluorescent cycle sequencing (ABI) (by Primm, Italy).

Sequence Analysis

We used a BLAST search (Altschul et al., '97) to identify sequences related to *RlDazl* and *RlPum1*. Sequence alignments were performed using MAFFT (Katoh et al., 2005). Phylogenetic trees were built using the Neighbor-joining method implemented in the MEGA 4.0 package (Tamura et al., 2007). Sequences were obtained from GenBank through the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov).

RNA Extraction and RT-PCR Analysis

Total RNA was extracted from different fresh or frozen adult tissues, as previously described. First strand cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen, Italy), from 1 µg of total RNA. RT-PCR analysis was performed using gene-specific sets of primers. β-*Actin* primers were used for standardization, as described by Marracci et al. (2007). For control reactions, reverse transcriptase was omitted.

In Situ Hybridization

Whole-mount in situ hybridizations were carried out on oocytes at various stages of maturity, from *P. ridibundus*, *P. lessonae*, and *P. esculentus*, using digoxigenin-labeled antisense and sense RNA probes generated from the full-length clones (cf. Ikenishi and Tanaka, 2000). Paraffin-embedded in situ hybridized oocytes were cut into 12 µm sections with a microtome. For in situ hybridization on cryostat sections (8–10 µm), testes and ovaries were fixed in 4% paraformaldehyde at room temperature for 2 hr, cryoprotected with 30% sucrose in PBS overnight at 4°C, and stored at −80°C until cryosectioning. Both testis and ovary cryosections were hybridized, as described by Marracci et al. (2007).

Immunofluorescence Reactions With Serine-10 Phosphorylated H3 Histone Antibody

Phosphorylated H3 histone was used as a marker of mitotic and meiotic prophase and metaphase. Immunofluorescence experiments with antibody against Ser-10 phosphorylated H3 histone have been carried out on sections of adult testis previously in situ hybridized, using methods described by Marracci et al. (2007).

RESULTS

Isolation of *RlDazl* and *RlPum1*

We isolated from *P. lessonae* full-length cDNA several clones, some homologous to *DAZL* and others to *Pumilio1*; and these were named *RlDazl* (GenBank accession no. AM490198) and *RlPum1* (GenBank accession no. FN547888), respectively. A phylogenetic analysis of *DAZL* proteins from several vertebrates confirmed that *RlDazl* belongs to the *DAZ* gene family (Fig. 1A). Interestingly, the *DAZL* gene seems to evolve faster in anurans than in other vertebrates (Fig. 1A). The 849 bp long sequence of *RlDazl* encodes a predicted protein of 282 amino acids that shares 92% amino acid identity with *Lithobates* (*Rana*) *pipiens* RpDazl, although it showed less than 60% identity with DAZL proteins of other vertebrates, including *Xenopus* (Table 1). The predicted RlDazl protein sequence contains a conserved RNA recognition motif (RRM) and a single DAZ motif (Fig. 1B). The *RlPum1* clone is 3,728 pb long and encodes for a predicted protein of 1,228 amino acids. The evolutionary tree of vertebrate *Pumilio* genes indicates that *RlPum1* belongs to the *Pumilio1* gene family (Table 2; Fig. 1C). The RlPum1 protein shares a very high sequence identity (>85%) with Pumilio1 proteins from other vertebrates, especially with other anuran Pumilio1 proteins (Table 2). The RlPum1 predicted amino acid sequence contains the PUM-HD domain, including the *N*-terminal conserved region, eight tandem imperfect Puf repeats, and the *C*-terminal region (Fig. 1D). The PUM-HD region shows the highest level homology (>90%) with other vertebrate species (data not shown).

Spatial Expression of *RlDazl* and *RlPum1* in Different *Pelophylax* Tissues

The expression pattern of these genes was investigated by RT-PCR on both somatic and gonadal tissues and showed that *RlDazl* was exclusively expressed in gonadal tissues, similar to *DAZL* genes characterized in other organisms (Fig. 2A). *RlPum1* is expressed not only in germline tissues, but also in somatic tissues, such as heart and spleen (Fig. 2B). The expression pattern of these genes seemed to be conserved in *P. lessonae, P. ridibundus*, and *P. esculentus* (data not shown).

Expression Profile of *RlDazl* and *RlPum1* During *Pelophylax* Oogenesis

In situ hybridization carried out on both juvenile and adult ovaries of *P. ridibundus, P. lessonae*, and *P. esculentus* females showed that *RlDazl* and *RlPum1* were expressed at early stages of oogenesis, with distinct profiles of mRNA distribution along the A/V oocyte axis. *RlDazl* is distributed throughout the cytoplasm of some but not all pre-vitellogenic stage I oocytes (Fig. 3a). Hoechst staining of hybridized sections, obtained from immature ovaries of metamorphosed froglets, highlighted the presence of the *RlDazl* transcript in oocytes at the beginning of meiotic prophase I (Fig. 3b and c). The intensity of the hybridization signal increases in the cytoplasm of oocytes I as meiotic prophase

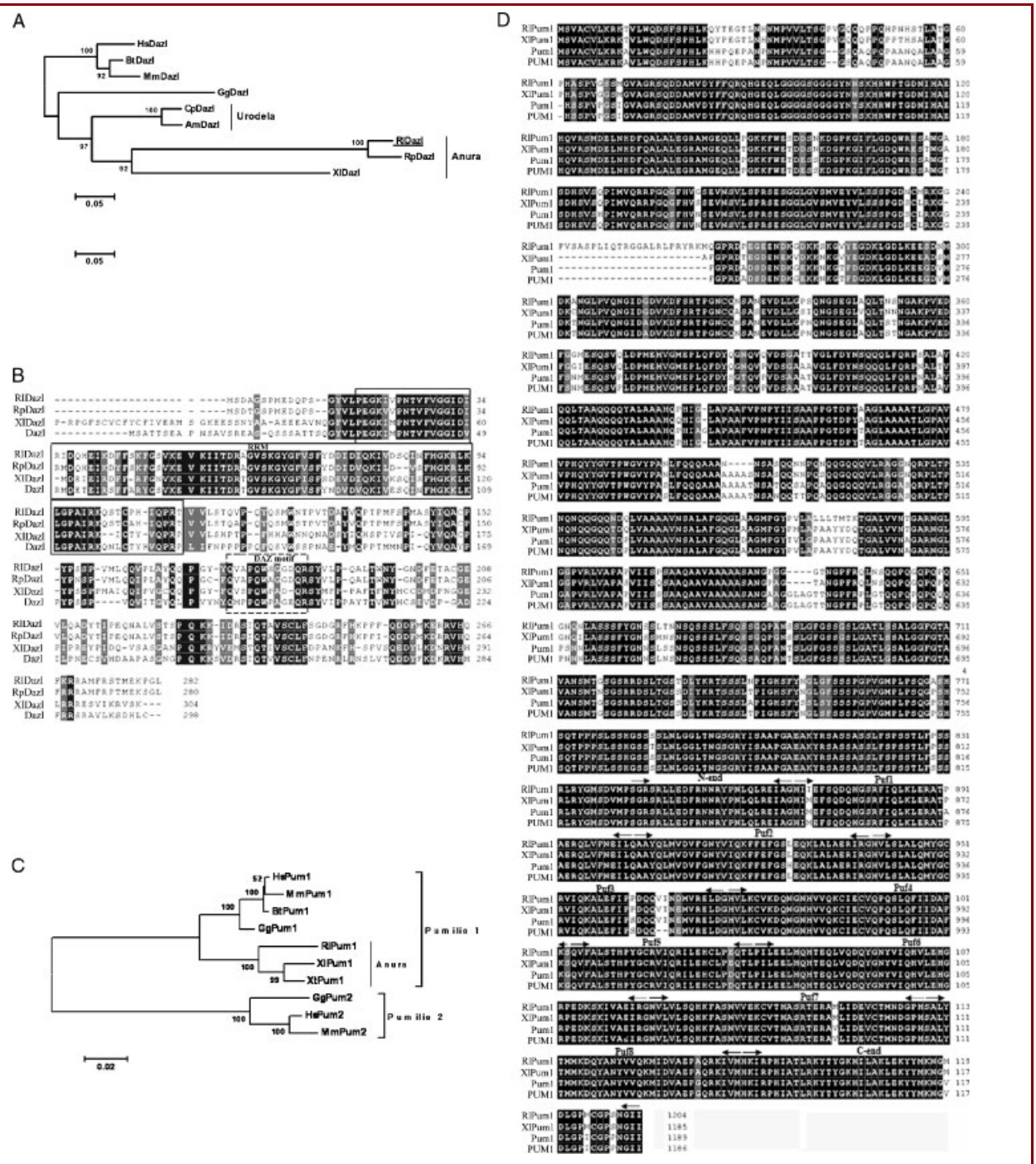

Figure 1. Sequence, structure, and phylogeny of RlDazl and RlPum1 proteins. Phylogenetic trees of Dazl proteins (**A**) and Pumilio1 family proteins (**C**) from the alignment of amino acid sequences. Neighbor-joining algorithm, 1,000 bootstrap replicates. Abbreviations correspond to species names shown in Tables 1 and 2. (**B**) Amino acid sequence alignment of Dazl proteins of *P. lessonae* (RlDazl; AM490198), *L. pipiens* (RpDazl; AAV30542), *X. laevis* (*XlDazl* l; AAH97658), and mouse (Dazl; NP_034151). Identical amino acids are in black, conservative substitutions are in gray. The RNA recognition motif (RRM) and DAZ motif are highlighted in frame and are boxed with continuous and dotted lines, respectively. (**D**) Alignment of the deduced amino acid sequences of the Pumilio proteins of *P. lessonae* (RlPum1; FN547888), *X. laevis* (XlPum1; BAC57980), mouse (Pum1; AAG42319), and *Homo sapiens* (PUM1; NP_055491). Identical amino acids are in black, conservative substitutions are in gray.

Table 1. Identity of RpDazl to Dazl proteins from other vertebrates.

Protein	Protein length	% Identity	Coverage	Accession number
RpDazl	280	92	100	AY645797.1
XlDazl	286	57	91	BC071023.1
CyDazl	302	56	97	AB164065.1
AxDazl	266	57	93	AF308872.1
GgDazl	289	53	92	NM_204218.1
HsDazl	295	56	92	U66726.2
MmDazl	298	56	92	NM_010021.4
BtDazl	295	58	92	EF501823.2

Coverage indicates the proportion of RpDazl that aligned with each Dazl protein. Rp, *Rana pipiens* (Anura); Xl, *Xenopus laevis* (Anura); Cy, *Cynops pyrrhogaster* (Urodela); Ax, *Ambystoma mexicanum* (Urodela); Gg, *Gallus gallus*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Bt, *Bos taurus*.

Table 2. Identity of RpPum1 to Pumilio proteins from other vertebrates.

Protein	Protein length	% Identity	Coverage	Accession number
XlPum1	1,185	93	100	CU075675.1
XtPum1	1,185	92	100	AB091091.1
GgPum1	1,189	87	100	DQ275191.1
HsPum1	1,188	87	100	NM_001020658.1
MmPum1	1,188	86	100	NM_001193123.1
BtPum1	1,186	86	100	BC048174.1
GgPum2	1,061	74	89	NM_001039292.1
HsPum2	1,064	73	89	NM_015317.1
MmPum2	1,066	72	89	NM_030723.2

Coverage indicates the proportion of RpPum1 that aligned with each Pum protein. Xl, *Xenopus laevis* (Anura); Xt, *Xenopus tropicalis* (Anura); Gg, *Gallus gallus*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Bt, *Bos taurus*.

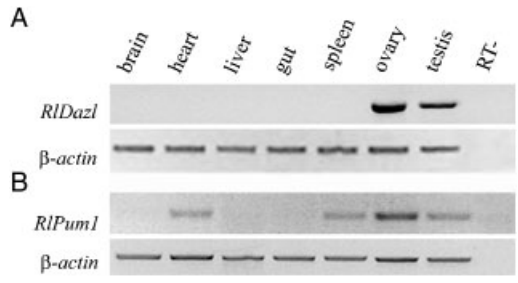

Figure 2. RT-PCR analysis of the expression pattern of *RlDazl* (A) and *RlPum1* (B) in adult tissues of *P. lessonae*; RT is the negative control and β-*actin* was used as a positive control.

progresses. From stage II, the *RlDazl* transcript has been found progressively accumulated at the vegetal pole of oocytes, and in later stages of oogenesis (III–VI) it has been detected in the subcortical region of the vegetal pole (Fig. 3d–j). This pattern was consistently observed in *P. ridibundus*, *P. lessonae*, and the *P. esculentus*.

The *RlPum1* gene is expressed in early stages of oogenesis (Fig. 4a). In situ hybridization on sections revealed a strong signal uniformly diffused through the cytoplasm of stage I oocytes (Fig. 4b). Unlike *RlDazl*, *RlPum1* mRNA has been detected preferentially located at the animal pole of oocytes from stage II of oogenesis in *P. ridibundus*, *P. lessonae*, and *P. esculentus* (Fig. 4c; data not shown). Neither gene was expressed in ovarian somatic cells (data not shown).

Expression Profile of *RlDazl* and *RlPum1* During *Pelophylax* Spermatogenesis

In males of the *P. esculentus* complex, the seminiferous tubules contain groups or cysts of spermatogenic cells synchronously differentiating (Ogielska and Bartmańska, '99). In situ hybridizations on adult testis sections of *P. ridibundus*, *P. lessonae*, and *P. esculentus* revealed that both *RlDazl* and *RlPum1* are expressed in single primary spermatogonia and groups of primary spermatocytes, whereas their mRNAs are absent in secondary spermatocytes, spermatids, and spermatozoa (Fig. 5a and d). Using serine-10 phosphorylated H3 as marker of meiotic division, we observed that both genes share the highest expression signal in nondividing primary spermatocytes, whereas the signal decreased as they entered in prophase-I and then disappeared in subsequent spermatogenic stages, such as secondary spermatocytes, spermatids, and spermatozoa (Fig. 5b and c). Hoechst staining of the hybridized sections have been used for recognizing spermatogenic cell types (Fig. 5c and f). Neither of the two genes was expressed in somatic cells of the testis (data not shown).

DISCUSSION

We have isolated from species of *Pelophylax* genes homologous to *Dazl* and *Pumilio1* and examined their expression patterns. Both genes encode for proteins with conserved RNA-binding motifs. Like *DAZL* genes characterized in other species, *RlDazl* showed an RRM and a single DAZ motif that has been demonstrated to be involved in multiple protein–protein interactions (Moore et al., 2003). *RlPum1* revealed a highly conserved PUM-HD region shared by all the other members of the *Pumilio* family. The crystal structures of *Drosophila* Pumilio and human PUM-HD domain have revealed that the Puf repeats are aligned in tandem to form an extended curved molecule (Wang et al., 2002). The RNA binds to the concave surface of the molecule, where each of the eight repeats makes contact with a different RNA base via three conserved amino acid residues positioned in the middle of the repeats (Wang et al., 2002). The presence of

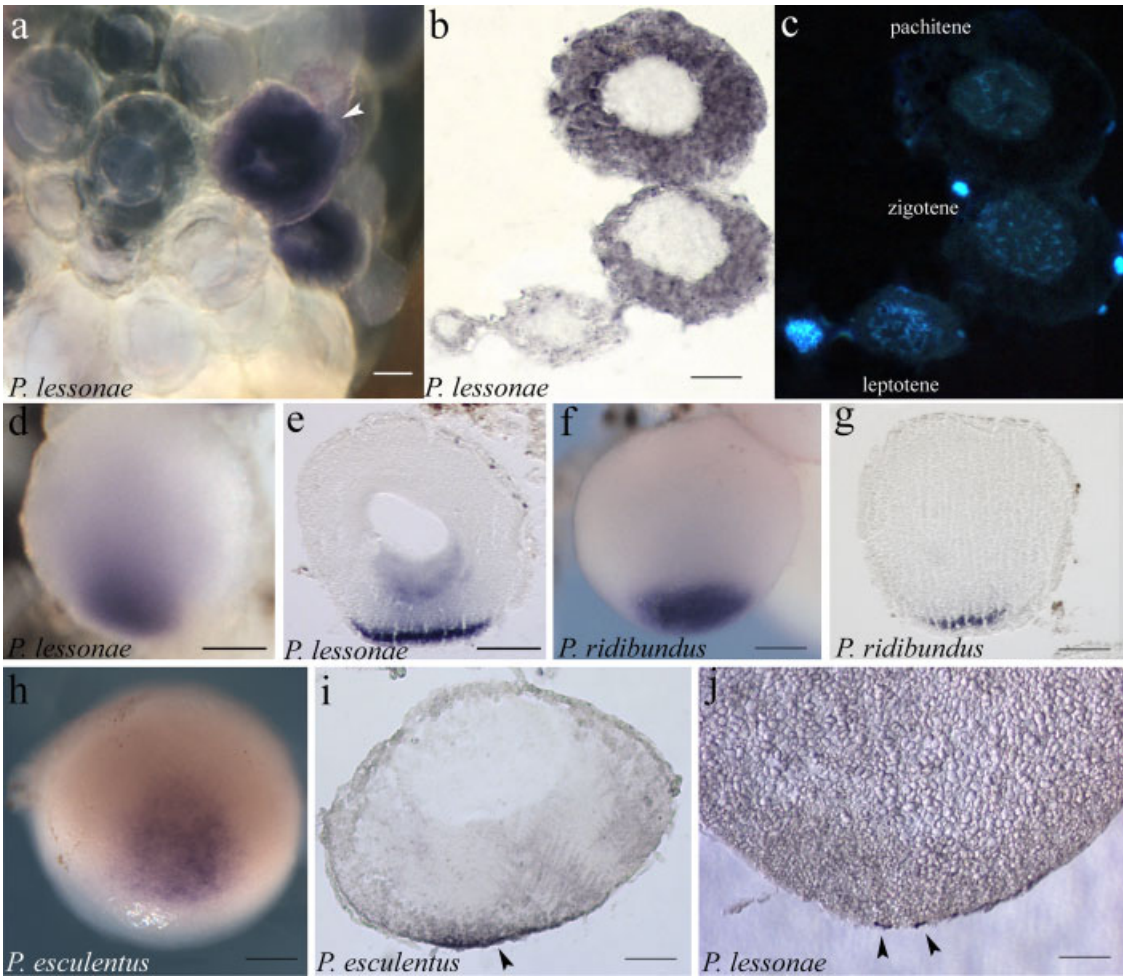

Figure 3. Expression pattern of *RlDazl* during oogenesis. Whole-mount in situ hybridization showing the localization of the *RlDazl* transcript in some pre-vitellogenic stage I oocytes of *P. lessonae* (**a**); sections from immature ovaries hybridized with *RlDazl* probe (**b**) and Hoechst staining of the hybridized section (**c**). Whole-mount in situ hybridization (**d**, **f**, **h**) in oocyte stage II (d), late stage II (f), stage III (h) with *RlDazl* probe of both the parental species and hybrid. Paraffin sections of whole-mount in situ hybridized oocytes (**e**, **g**, **i**) and of oocyte stage VI (**j**) showed that the *RlDazl* transcript progressively accumulated at the vegetal pole (downwards). The white arrowhead points to a pre-vitellogenic oocyte; the black arrowheads indicate *RlDazl* transcript detected in the subcortical region of the vegetal pole. Scale bars represent 50 μm (a, b); 100 μm (d–j).

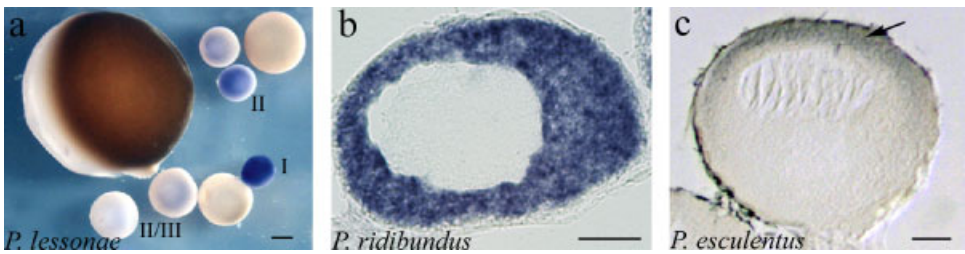

Figure 4. Expression pattern of *RlPum1* during oogenesis. (**a**) Whole-mount in situ hybridization showed a strong signal in stage I oocytes. This signal declined in oocyte stages II and III. (**b**) Paraffin section of whole-mount in situ hybridized stage I oocyte showed that *RlPum1* mRNA was widely distributed in the ooplasm. (**c**) Paraffin sections of whole-mount in situ hybridized stage III oocyte shows that the *RlPum1* transcripts are located at the animal pole (upwards). Scale bars represent 400 μm (a); 25 μm (b); 100 μm (c).

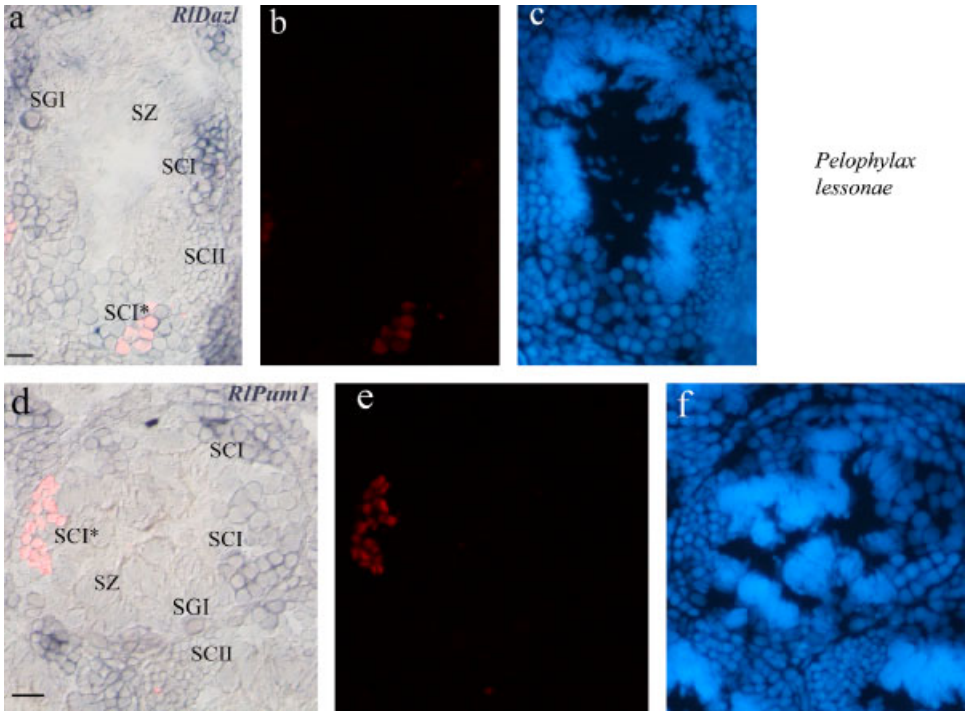

Figure 5. *RlDazl* and *RlPum1*expression in the testis. (a) Section of adult testis of *P. lessonae* hybridized with *RlDazl* probe (blue signal); (b) Same hybridized section immunostained with anti-phosphohistone H3 expression (red signal) and counterstained with Hoechst (c); (d) *Rlpum1* hybridization signal (in blue); (e) same hybridized section immunostained with anti-phosphohistone H3 (red signal) and counterstained with Hoechst (f). SGI, primary spermatogonia; SCI, primary spermatocytes; SCII, secondary spermatocytes; SZ, spermatozoa. The asterisks denote primary spermatocytes undergoing meiotic division. Scale bars represent 250 μm.

highly conserved RNA-binding domains suggested for these two genes a possible role as translational regulators.

The spatial expression analysis performed on different adult tissues showed that *RlDazl* is exclusively expressed in germ line tissues, whereas the *RlPum1* transcript has been also detected in some somatic tissues. Both *RlDazl* and *RlPum1* were expressed during the early oogenesis, revealing a differential mRNA distribution along the A/V axis of the oocyte. Because maternally localized mRNAs are differentially segregated into blastomeres during early development, the unequal distribution of *RlDazl* and *RlPum1* mRNAs along the A/V axis suggests that the two genes differentially contribute to the embryo axis formation as well as the specification of the cell fate. The *RlPum1* transcript was preferentially enriched at the animal hemisphere of oocytes of *P. ridibundus*, *P. lessonae*, and *P. esculentus*. Some localized mRNAs so far identified are enriched at the animal pole, but their developmental significance remains to be demonstrated (Schnapp et al., '97; Mowry and Cote, '99; King et al., 2005).

Like *XlDazl* of *X. laevis* and *RpDazl* of *L. pipiens*, the *RlDazl* transcript, from stage II of oogenesis, was found progressively localized to the oocyte vegetal pole. Molecular studies carried out in *Xenopus* highlighted that some vegetally localized RNAs are involved in germ cell specification (Nakahata et al., 2001, 2003); *RlDazl* may play an analogous role in these water frogs. The expression of *RlDazl* is variable in stage I oocytes, just as is the expression of *XlDazl* (Houston et al., '98) and *RpDazl* (Nath et al., 2005). In particular, the intensity of the hybridization signal of *RlDazl* increased in the cytoplasm of stage I oocytes as the meiotic prophase progresses, suggesting that *RlDazl* expression could be regulated in oocytes advancing through meiosis. Interestingly, Haston et al. (2009) showed that, in *DAZL*-null mice, female germ cells fail to progress through meiosis. In *C. elegans* the DAZ-1 protein plays an essential role at premeiotic and early meiotic stages in female germ cells and facilitates the proper progression of oogenesis (Maruyama et al., 2005). In the oocytes of the urodele amphibians *Cynops pyrrhogaster* and *Ambystoma mexicanum* (axolotl), the *Cydazl* and *Axdazl* mRNAs, respectively, show no specific localization in the ooplasm, which is consistent with there being no germ plasm (Bachvarova et al., 2004; Tamori et al., 2004).

RlDazl and *RlPum1* showed a similar expression pattern during spermatogenesis. Both genes are expressed in spermatogonia and primary spermatocytes entering into meiosis. A similar pattern was shown by *Xenopus XlDazl*. The authors demonstrated

that *XlDazl* can rescue meiotic entry of spermatocytes in *Drosophila Boule* mutants, suggesting a possible role of this gene as regulator of meiotic division (Houston et al., '98). In mouse models, the absence of the autosomal *DAZL* gene resulted in a final block at zygotene of meiotic prophase (Reynolds et al., 2005, 2007). The *C. elegans pumilio* homolog *puf-8* is required for a normal progression of meiosis of primary spermatocytes and in *puf-8* mutants, primary spermatocytes dedifferentiate into mitotic germ cells (Subramaniam and Seydoux, 2003).

The genome exclusion and genome endoreduplication typical of the *Pelophylax* hybridogenesis are believed to occur only during the germline development in the hybrids. Given such a massive reorganization of the germline genome, one would expect to find substantial differences during the gametogenesis of the hybrids when compared with the parental species. We have shown in this article that the expressions pattern of *RlDaz1* and *RlPum1* during the gametogenesis does not differ among *R. ridibundus*, *R.lessonae*, and *R. esculentus*. These results mirror our previously reported molecular analyses of the expression pattern of other genes (Marracci et al., 2007, 2008), and suggest that despite the remarkable changes in the hybrids germline associated with the hybridogenesis (cf. Ogielska and Kotusz, 2004), both oogenesis and spermatogenesis follow similar genetic pathways of differentiation in adults of hybrids and parental species.

In addition, we noticed strong similarities in the expression pattern of *DAZL* during the gametogenesis of *Pelophylax* and *Xenopus* species. Since the families Ranidae, which includes *Pelophylax*, and Pipidae, comprising *Xenopus*, diverged about 230 million years ago (Roelants et al., 2007), our results indicate that the expression pattern of genes involved in the gametogenesis is highly conserved between these distantly related anurans. Intriguingly, the consistent expression pattern of *DAZL* in *Pelophylax* and *Xenopus* is not reflected at the protein sequence level, as we observed an accelerated evolution of this gene in anurans compared with other vertebrates.

RlDazl joins *RlVlg* and *RlYb2* (Marracci et al., 2007, 2008), as the only specific markers of germ cells so far known in water frogs. These genes constitute distinctive markers of specific phases of oogenesis and spermatogenesis, useful in order to explore the hybridogenetic process in these water frogs.

Overall, these findings point to the relevance of the *Pelophylax* group of water frogs as a model system complementing our knowledge in the development and maturation of the germline derived from studies on other vertebrates, and highlight the importance of gene expression analyses in the study of the hybridogenetic gametogenesis.

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